http://dx.doi.org/10.1080/19440049.2014.1003336

Streptomyces–Aspergillus flavus interactions: impact on aflatoxin B accumulation

C. Verheecke, T. Liboz, P. Anson, Y. Zhu and F. Mathieu*

Laboratoire de Génie Chimique, Université de Toulouse, Castanet-Tolosan, France

The aim of this work was to investigate the potential of *Streptomyces* sp. as biocontrol agents against aflatoxins in maize. As such, we assumed that *Streptomyces* sp. could provide a complementary approach to current biocontrol systems such as Afla-guard® and we focused on biocontrol that was able to have an antagonistic contact with *A. flavus*. A previous study showed that 27 (out of 38) *Streptomyces* sp. had mutual antagonism in contact with *A. flavus*. Among these, 16 *Streptomyces* sp. were able to reduce aflatoxin content to below 17% of the residual concentration. We selected six strains to understand the mechanisms involved in the prevention of aflatoxin accumulation. Thus, in interaction with *A. flavus*, we monitored by RT-qPCR the gene expression of *aflD*, *aflM, aflP*, *aflR* and *aflS*. All the *Streptomyces* sp. were able to reduce aflatoxin concentration (24.0–0.2% residual aflatoxin B_1). They all impacted on gene expression, but only S35 and S38 were able to repress expression significantly. Indeed, S35 significantly repressed *aflM* expression and S38 significantly repressed *aflR*, *aflM* and *aflP*. S6 reduced aflatoxin concentrations (2.3% residual aflatoxin B_1) and repressed *aflS*, *aflM* and enhanced *aflR* expression. In addition, the S6 strain (previously identified as the most reducing pure aflatoxin B_1) was further tested to determine a potential adsorption mechanism. We did not observe any adsorption phenomenon. In conclusion, this study showed that *Streptomyces* sp. prevent the production of (aflatoxin gene expression) and decontamination of (aflatoxin B_1 reduction) aflatoxins *in vitro*.

Keywords: *Aspergillus flavus*; *Streptomyces*; co-culture; degradation; RT-qPCR; gene expression; aflatoxin

Introduction

Aflatoxins B_1 and B_2 (AFBs) are secondary metabolites produced by filamentous fungi. The former are polyketide-derived furanocoumarins. Because of their carcinogenicity (IARC 2014) their occurrence in food and feed is a major food-related health issue. Thus, aflatoxins are regulated in maize from 4 µg kg^{-1} in the European Union and at levels up to 20 µg kg^{-1} in China. Among the producing fungi, *Aspergillus flavus* is the most common in different crops including maize, hazelnuts, peanuts, etc. (Giorni et al. 2007; Passone et al. 2010). Aflatoxin contamination in maize (our targeted commodity) has already been well studied. Abiotic (temperature, water activity (a_w), pH etc.) and biotic parameters can prevent aflatoxin accumulation (Holmquist et al. 1983; Keller et al. 1997; Wilkinson et al. 2007; Holmes et al. 2008). In this paper we have studied biotic solutions at maize field level.

There are currently biocontrol measures to prevent aflatoxin accumulation such as Afla-guard® (USA) and Afla-safe® (Africa). With these treatments non-aflatoxigenic *A. flavus* overtake the maize fungal niche and prevent other mycotoxigenic fungi colonisation. The later prevents aflatoxin occurrence up to 70.1–99.9% (Atehnkeng et al. 2008). Other microorganisms potentially inhibit aflatoxin accumulation (e.g. *Fusarium* spp. and *Streptomyces* spp.). These examples reduce the AFB1 accumulation by up to 93–96% on peanut and maize grain (Marín et al. 2001; Zucchi et al. 2008).

A previous study already investigated the interaction between actinomycetes isolates and *A. flavus*. After a 10-day co-incubation *in vitro*, 27 isolates showed mutual antagonism in contact with the 37 actinomycetes isolates tested. Moreover, 16 isolates reduced the AFB1 residual concentration below 17%. Among them, 12 isolates were tested for their ability to reduce pure AFB1 content. After 4 days at 28°C on ISP-2 medium, AFB1 (5 mg kg^{-1}) was reduced by eight isolates. The remaining AFB1 concentration varied between 82.2% and 15.6% (Verheecke et al. 2014).

In terms of curative approach, biotic stimuli could also act directly on aflatoxin molecules to reduce concentrations. Indeed, bacteria were shown to detoxify, adsorb or degrade AFB1 (Alberts et al. 2009; Wu et al. 2009).

In order to understand the mechanisms involved in the prevention of aflatoxin accumulation, we proposed monitoring the expression of targeted aflatoxin genes. Indeed, 30 putative genes constitute the cluster (80 kb) coding for the aflatoxin molecular pathway (Yu 2012). AflS is a co-activator of AflR: a transcription factor fixing a consensus sequence localised in aflatoxin gene promoters (Payne et al. 1993; Meyers et al. 1998). Concerning structural genes, the most studied are *aflD*, *aflM* and *aflP*, encoding a norsolorinic acid reductase, a versicolorin

*Corresponding author. Email: mathieu@ensat.fr

A deshydrogenase and a sterigmatocystin methyltransferase, respectively (Papa 1982; Bhatnagar et al. 1988; Skory et al. 1992).

As *Streptomyces* are potential biocontrol agents, it is crucial to understand their mechanism of action prior to *in vivo* testing. Thus, thanks to different techniques, we investigated *Streptomyces* sp. effects on the prevention or reduction of concentrations of aflatoxins.

Materials and methods

Fungal strain and actinomycete isolates

The fungal strain used was *A. flavus* NRRL 62,477. *Streptomyces* isolates (collected from Algerian soils) were macroscopically observed and validated as belonging to the *Streptomyces* genus. The isolates with the less antagonistic characteristics versus *Aspergillus* sp. were selected (Verheecke et al. 2014). They were stored at −20°C in cryotubes in a 20% glycerol solution in our laboratory.

Interaction method

The interaction methodology was carried out as described elsewhere (Verheecke et al. 2014), with slight modifications. A sterile 8.5 cm cellophane sheet (Hutchinson, Chalette-sur-loing, France) was added to the Petri dish containing the ISP2 medium. *A. flavus* inoculation (centre of the Petri dishes, 10 µl of 10^6 spores ml^{-1}) and *Streptomyces* (two streaks) were simultaneously inoculated 2 cm away. The fungal biomass with the cellophane, without bacterial biomass (scalpel cut), was removed from the cellophane surface after 90 h of incubation at 28°C. The experiments were carried out twice in triplicate.

Aflatoxin extraction and quantification

Aflatoxin extraction and quantification was carried out as previously described (Verheecke et al. 2014). Briefly, agar plugs were taken (approximately 1 g) within the fungal area. Aflatoxins were extracted by methanol addition (1 ml) followed by a 30 min incubation period (shaken three times). Then, the extract was centrifuged for 15 min at 12 470*g* and the supernatant was filtered (0.45 µm, 4 mm PVDF; Whatman, Maidstone, UK) into vials. Quantification of aflatoxins was carried out on an Ultimate 3000 system (Dionex-Thermo Electron, Orsay, France) with all the RS series modules. A C-18 pre-column and C-18 column were used (Phenomenex, Le Pecq, France; Luna 3 µm, 200 × 4.6 mm). Aflatoxin detection was carried out according to the Coring Cell® instruction (Coring System Diagnostix GmbH, Gernsheim, Germany). Quantification was undertaken with Chromelon software, using standards of AFB1 and AFB2 (Sigma-Aldrich, Saint-Quentin-Fallavier, France) (detection limit = 0.5 ng g^{-1}). Statistics were made with 'nparcomp' R (2.15.2).

RNA extraction, RT and qPCR

The fungal biomass was ground in liquid nitrogen and stored at −80°C. Approximately 60 mg of mycelia were taken for RNA extraction. Total RNA was isolated using the Aurum Total RNA Kit (BioRad, Marnes-la-Coquette, France) according to the manufacturer's instructions for eukaryotic and plant cell material with the following modifications: DNase I digestion increased to 1 h and the elution was carried out at 70°C for 2 min in elution buffer. RNA quantity and quality were checked by Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA) and Experion (BioRad) according to manufacturers' instructions.

RT was carried out with the Advantage RT-PCR Kit (Clontech, MountainView, CA, USA) with Oligo $(dT)_{18}$ primer according to the manufacturer's instructions with one modification: reaction incubation at 42°C was increased to 4 h. RT-qPCR are performed in duplicate in a CFX96 Touch instrument (Bio-Rad) using SsoAdvanced™ SYBR Green Supermix (Bio-Rad). Following the RT-qPCR, data were analysed using CFX Manager Software (version 3.0, Bio-Rad). Statistics were carried out with qbase+ software (biogazelle) with *act1* and *βtub* as reference genes and a one-way analysis of variance (ANOVA) (control versus all strains), paired *t*-test (control versus each strain).

Aflatoxin B_1 adsorption test

S13 and S6 spores were dislodged from the pre-culture with a sterile loop and placed in 10 ml sterile water. Spores and AFB1 were added in a glass vial with a final concentration of 10^6 spores ml^{-1} and 1 µg ml^{-1}, respectively. Spores were removed by filtration (PVDF, 13 mm, 0.45 µm; Whatman). After 1 or 60 min incubation at 30°C, the remaining solution was transferred into vial number 1. The filter was rinsed twice: sterile water (1 ml) and methanol; the rinse liquids were dropped off in vial numbers 2 and 3, respectively. The experiment was carried out twice in triplicate. A Student's *t*-test was applied for statistical analysis.

Results

Interaction of Streptomyces–Aspergillus flavus

The six *Streptomyces* strains had mutual antagonism on contact with *A. flavus* and the aflatoxin accumulation (about 500 ng g^{-1} for AFB1 and approximately 50 ng g^{-1} for AFB2 in the control) was highly reduced during interactions. S17, S13 and S27 showed the lowest reduction with 24%, 15.6% and 8.1% AFB1 residual concentration in the media (rcm), respectively. S38, S6 and S35 were more efficient with 3.1%, 2.3% and 0.2% rcm AFB1, respectively. In order to understand if these strains can prevent aflatoxin accumulation, we decided to study the

interaction effect on gene expression with a RT-qPCR approach.

RT-qPCR aflatoxin gene expression

We studied the expression of some aflatoxin genes within *A. flavus* alone (control) and in interaction with six different *Streptomyces* strains. Five genes (*aflD*, *aflM, aflP*, *aflR* and *aflS*) were chosen for their relative expression. Table 1 summarises the gene expression normalised with the controls. *aflD* expression was not significantly impacted in all tested conditions. Only S35 and S38 significantly repressed gene expression. Both strains repressed a*flM* expression by more than seven-fold. Moreover, S38 significantly repressed *aflP* expression 4.8-fold and *aflR* expression 1.4-fold.

Effects of selected actinomycetes isolates on pure AFB1

A previous study showed that some of those strains can impact AFB1 concentration (5 mg kg^{-1}) in ISP2 medium. Briefly, S6 was the most efficient with an rcm of 15.6%. S27, S38 and S35 showed a significant reduction in AFB1 concentration with 76.6%, 38.0% and 29.4% rcm, respectively; and S13 and S17 showed no significant impact. S6 (most efficient) and S13 (negative control) were further tested for potential adsorption capacities. The results are presented in Table 2. At both incubation times (1 and 60 min), the recoveries from the supernatant, rinse water and rinse methanol were not different from the control. Those results bring out the potential absence of binding in the S6's AFB1 reducing process.

Conclusions

The tested *Streptomyces* strains reduce AFBs' accumulation in interaction with *A. flavus.* There were two different patterns concerning AFBs' accumulation. The first pattern: S6, S35 and S38 highly reduced AFBs' rcm in Petri dish co-culture and highly removed pure AFB1 in the medium. Further investigation showed that S6 was not able to bind AFB1. Regarding gene expression, S6 repressed *aflS* ($p < 0.19$) and *aflM* ($p < 0.19$), S35 and S38 repressed *aflM* and *aflR* ($p < 0.09$ and < 0.08, respectively), and S38 repressed *aflP*. The second pattern – S13, S17 and S27 – also reduced AFBs' rcm but was less efficient in pure AFB1 removal. This pattern showed no significant impact on gene expression.

Table 1. Results concerning aflatoxin accumulation and gene expression by six chosen *Streptomyces* strains.

	Effect on AFB accumulation in co-culture		Effect on gene expression				
Strain	Aflatoxin B1 (% rcm)	Aflatoxin B2 (% rcm)	*aflR*	*aflS*	*aflD*	*aflM*	*aflP*
Control	100.5 ± 5.5[a]	100.9 ± 9.4[a]	1.00	1.00	1.00	1.00	1.00
S6	2.3 ± 4.5[c]	n.d.	2.37	0.40	0.69	0.25	1.57
S13	15.6 ± 9.2[b]	9.3 ± 20.8[b]	0.82	0.70	1.60	0.45	0.41
S17	24.0 ± 19.8[b]	5.3 ± 11.9[b]	1.53	0.39	0.95	0.26	3.03
S27	8.1 ± 5.1[b]	n.d.	0.88	0.96	1.42	0.26	0.39
S35	0.2 ± 0.5[c]	n.d.	0.63	0.24	0.50	0.12*	1.02
S38	3.1 ± 5.3[c]	n.d.	0.69*	0.62	1.44	0.14*	0.21*

Note: Data with the same letter are not significantly different ($p < 0.05$). *Significant difference ($p < 0.05$). rcm, Residual concentration in the media.

Table 2. Adsorption test results.

	Incubation time: 1 min			
Strain/vial	1	2	3	Total recovery (%)
Control	73.5 ± 6.8	14.2 ± 2.0	5.9 ± 3.0	93.6 ± 11.8
S6	81.3 ± 12.7	15.3 ± 1.9	4.4 ± 0.5	101.1 ± 15.2
S13	80.8 ± 9.1	15.7 ± 2.0	4.8 ± 1.2	101.3 ± 12.3
	Incubation time: 60 min			
Control	72.7 ± 10.3	13.7 ± 1.0	3.8 ± 0.8	90.2 ± 12.1
S6	71.0 ± 6.9	14.1 ± 1.9	4.2 ± 0.9	89.2 ± 9.7
S13	81.1 ± 8.3	14.3 ± 2.0	3.4 ± 0.5	98.8 ± 11.5

Notes: Cells were suspended in water in the presence of AFB1 (1 µg) and incubated at 30°C for 1 and 60 min. Data are means ± standard deviations (SDs) as a percentage of the standard. 1, Supernatant; 2, rinse water; and 3, rinse methanol.
No data were significantly different according to the *t*-test ($p < 0.05$).

Discussion

Few studies have focused on *Streptomyces–Aspergillus* interactions. Usually scientists have chosen the inhibition of fungal growth as the first required step for biocontrol selection (Sultan & Magan 2011; Haggag & Abdall 2012). Our team worked on the promotion of growth of microorganisms and reduction of aflatoxin concentrations. We previously showed that actinomycetes isolates can have mutual antagonism in contact and reduce AFB1 residual concentration under 17% in interaction with *A. flavus* (Verheecke et al. 2014).

Here, we tested six of these strains for their ability to prevent aflatoxin accumulation. The co-culture results showed mutual antagonism on contact with *A. flavus*. S17, S13 and S27 showed the lowest aflatoxin reduction, while S38, S6 and S35 were more efficient. These results are in line with our previous data. Indeed, even if the co-culture conditions were modified (addition of a cellophane sheet, inoculation modification in time and space), similar results were obtained in both co-culture conditions. Thus, the results of this study confirm that six *Streptomyces* strains have the capacity to reduce aflatoxin accumulation *in vitro*.

Our *Streptomyces* strains can reduce aflatoxin accumulation in interaction with *A. flavus*. In 1997, Ono et al. (1997) identified *Streptomyces* sp. MRI142 as a producer of aflastatin A. This molecule (0.5 µg ml^{-1} in the medium) completely inhibited B1 production without impacting on fungal growth (Ono et al. 1997). Aflastatin A inhibition mechanisms were further investigated by RT-qPCR. At a 0%, 1% (v/v) concentration, aflastatin A inhibited the expression of *aflC*, *aflM*, *aflP* and *aflR* in *A. parasiticus* ATCC24690 (Kondo et al. 2001). In our study our strain S38 inhibited *aflM*, *aflP* and *aflR* expression. Remaining fungal growth, reduced aflatoxin concentrations and reduced gene expression suggest that aflastatin A could be produced by this strain.

S35 and S38 strains repressed *aflM* and *aflP* compared with the control. A possible mechanism involved in the S35 and S38 pattern could be linked to a modification in the presence of *laeA*. A gene mutation of *laeA* in *A. flavus* revealed a 100-fold less expression of *aflM* and *aflP* (Chang et al. 2012). Thus, a *laeA* repression could be involved in the reduction of aflatoxin production by S35 and S38.

An additional advantage to our biocontrol candidate could be pure AFB1 removal thanks to adsorption or degradation mechanisms. A previous study showed that S6, S35 and S38 were able to reduce greatly pure AFB1 concentrations (15.6%, 29.4% and 38% rcm, respectively). This mechanism could be linked to cell wall surface binding such as described in lactic acid bacteria (El-Nezami et al. 1998).

In our study, we investigated S6's capacity to bind pure AFB1 (1 µg ml^{-1}). The results showed that S6 cannot bind AFB1. Another possibility is the enzymatic degradation of AFB1. For example, $F_{420}H_2$ reductase is commonly found in *Actinomycetales* genus. The former transforms AFB1 into several small molecules (Taylor et al. 2010). Nevertheless, this reductase has not as yet been characterised in *Streptomyces* genus. S17, S27 and S13 are able to reduce AFBs' accumulation regardless of the studied mechanisms.

In conclusion, this study confirms the capacity of this six actinomycetal strains to reduce *in vitro* the accumulation of AFBs. S35 and S38 were the best repressors of gene expression, while S6 showed the best capacity to reduce pure AFB1 concentrations without binding. These three strains have to be further investigated in a greenhouse environment to evaluate their ability to maintain their interesting characteristics.

Acknowledgements

The authors would like to thank Lora Verheecke for her help.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the French National Research Agency [grant numbers 'Aflafree n°11 003 01'] and Agri Sud-Ouest Innovation.

References

Alberts JF, Gelderblom WCA, Botha A, van Zyl WH. 2009. Degradation of aflatoxin B1 by fungal laccase enzymes. Int J Food Microbiol. 135:47–52.

Atehnkeng J, Ojiambo PS, Ikotun T, Sikora RA, Cotty PJ, Bandyopadhyay R. 2008. Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Addit Contam: Part A. 25:1264–1271.

Bhatnagar D, Ullah AHJ, Cleveland TE. 1988. Purification and characterization of a methyltransferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway. Prep Biochem. 18:321–349.

Chang P-K, Scharfenstein LL, Ehrlich KC, Wei Q, Bhatnagar D, Ingber BF. 2012. Effects of laeA deletion on *Aspergillus flavus* conidial development and hydrophobicity may contribute to loss of aflatoxin production. Fungal Biol. 116:298–307.

El-Nezami H, Kankaanpaa P, Salminen S, Ahokas J. 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B1. Food Chem Toxicol. 36:321–326.

Giorni P, Magan N, Pietri A, Bertuzzi T, Battilani P. 2007. Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. Int J Food Microbiol. 113:330–338.

Haggag WM, Abdall AM. 2012. Evaluation of *Streptomyces Aureofaciens* and *Rhodotorulaglutinis* against ochratoxin A producing *Aspergillus nigri* in grapevines. J Microbiol Res. 2:170–175.

Holmes RA, Boston RS, Payne GA. 2008. Diverse inhibitors of aflatoxin biosynthesis. Appl Microbiol Biotechnol. 78:559–572.

Holmquist GU, Walker HW, Stahr HM. 1983. Influence of temperature, pH, water activity and antifungal agents on growth of Aspergillus flavus and A. parasiticus. J Food Sci. 48:778–782.

IARC. 2014. Monographs- classifications [Internet]. [cited 2012 May 14]. Available from: http://monographs.iarc.fr/ENG/Classification/index.php

Keller NP, Nesbitt C, Sarr B, Phillips TD, Burow GB. 1997. pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. Phytopathology. 87:643–648.

Kondo T, Sakurada M, Okamoto S, Ono M, Tsukigi H, Suzuki A, Nagasawa H, Sakuda S. 2001. Effects of aflastatin A, an inhibitor of aflatoxin production, on aflatoxin biosynthetic pathway and glucose metabolism in *Aspergillus parasiticus*. J Antibiot. 54:650–657.

Marín S, Albareda X, Ramos AJ, Sanchis V. 2001. Impact of environment and interactions of *Fusarium verticillioides* and *Fusarium proliferatum* with *Aspergillus parasiticus* on fumonisin B1 and aflatoxins on maize grain. J Sci Food Agr. 81:1060–1068.

Meyers DM, Obrian G, Du WL, Bhatnagar D, Payne GA. 1998. Characterization of *aflJ*, a gene required for conversion of pathway intermediates to aflatoxin. Appl Environ Microbiol. 64:3713–3717.

Ono M, Sakuda S, Suzuki A, Isogai A. 1997. Aflastatin A, a novel inhibitor of aflatoxin production by aflatoxigenic fungi J Antibiot. 50:111–118.

Papa KE. 1982. Norsolorinic acid mutant of *Aspergillus*. J Gen Microbiol. 128:1345–1348.

Passone MA, Rosso LC, Ciancio A, Etcheverry M. 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of *nor*-1 gene, and effects of storage conditions on aflatoxin production. Int J Food Microbiol. 138:276–281.

Payne GA, Nystrom GJ, Bhatnagar D, Cleveland TE, Woloshuk CP. 1993. Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. Appl Environ Microbiol. 59:156–162.

Skory CD, Chang PK, Cary J, Linz JE. 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Appl Environ Microbiol. 58:3527–3537.

Sultan Y, Magan N. 2011. Impact of a *Streptomyces* (AS1) strain and its metabolites on control of *Aspergillus flavus* and aflatoxin B1 contamination *in vitro* and in stored peanuts. Biocontrol Sci Techn. 21:1437–1455.

Taylor MC, Jackson CJ, Tattersall DB, French N, Peat TS, Newman J, Briggs LJ, Lapalikar GV, Campbell PM, Scott C, Russell RJ, Oakeshott JG. 2010. Identification and characterization of two families of $F_{420}H_2$-dependent reductases from *Mycobacteria* that catalyse aflatoxin degradation. Mol Microbiol. 78:561–575.

Verheecke C, Liboz T, Darriet M, Sabaou N, Mathieu F. 2014. *In vitro* interaction of actinomycetes isolates with *Aspergillus flavus*: impact on aflatoxins B1 and B2 production. Lett Appl Microbiol. 58:597–603.

Wilkinson JR, Yu J, Abbas HK, Scheffler BE, Kim HS, Nierman WC, Bhatnagar D, Cleveland TE. 2007. Aflatoxin formation and gene expression in response to carbon source media shift in *Aspergillus parasiticus*. Food Addit Contam. 24:1051–1060.

Wu Q, Jezkova A, Yuan Z, Pavlikova L, Dohnal V, Kuca K. 2009. Biological degradation of aflatoxins. Drug Metab Rev. 41:1–7.

Yu J. 2012. Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. Toxins. 4:1024–1057.

Zucchi TD, De Moraes LA, De Melo IS. 2008. Streptomyces sp. ASBV-1 reduces aflatoxin accumulation by aspergillus parasiticus in peanut grains. J Appl Microbiol. 105:2153–2160.